

Synergistic Antitumor Effect of Andrographolide and Cisplatin Through ROS-Mediated ER Stress and STAT3 Inhibition in Colon Cancer

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Abstract

Colon cancer is one of the most leading death-causing cancers in the world. Cisplatin has been widely used as the first-line treatment of cancer. However, its clinical application is limited by the side effects or acquired drug resistance. Hence, it is of vital clinical significance to develop novel agents that synergize with cisplatin and decrease its side effects. The aim of this study was to investigate whether Andrographolide (AP) synergistically potentiates the anti-tumor effect of cisplatin on colon cancer cells. Here, we found that AP synergizes with cisplatin in exerting anticancer activity in colon cancer cells. Further studies showed that AP potentiates cisplatin-induced endoplasmic reticulum stress and STAT3 inhibition through increasing intracellular ROS. Notably, pre-treatment of NAC, a ROS scavenger, reversed apoptosis induced by combined treatment of AP and cisplatin, while relieving the activation of endoplasmic reticulum stress as well as STAT3 inhibition. These findings indicated that ROS plays a pivotal role in mediating synergistic anticancer effects of AP and cisplatin on colon cancer cells. Overall, this study presents a potential new therapeutic strategy for the treatment of colon cancer.

Introduction

Colon cancer ranks third in morbidity and mortality among all tumors[1]. There are approximately 1.2 million new cases with colon cancer and 600,000 colon cancer deaths each year[2]. The morbidity of colon cancer generally increases with age. While colon cancer is more prevalent in developed countries, the risk of colon cancer in developing countries has been increasing rapidly[2]. The pathogenesis of colon cancer is a complex multifactorial process involving mutations in tumor suppressor genes and/or specific oncogenes. Common treatment options for colon cancer include surgery, chemotherapy and radiotherapy. Among them, chemotherapy is widely used in clinic to eliminate residual tumor cells for improving the overall survival of patients [3]. However, the clinical benefits of chemotherapy are limited due to its severe side effects as well as chemotherapeutic resistance[4].

Cisplatin (CDDP) belongs to a class of platinum-containing anti-cancer drugs which form a covalent crosslink to DNA[5]. CDDP was more commonly used as chemotherapeutic agents for colon cancer than 5-Fluorouracil[6]. However, the widespread resistance to CDDP renders malignant cells less susceptible to anti-proliferative and cytotoxic effects of the drug. Thus, most of colon cancer patients treated with CDDP are destined to experience therapeutic failure and tumor recurrence[7]. Recently, combined chemotherapy involving CDDP has been proved to be a better treatment strategy compared with single-agent therapy[8–10]. Therefore, it is urgently needed to search for effective agents that are capable of enhancing the therapeutic effect of CDDP and minimizing its side effects.

Reactive oxygen species (ROS) are generated during normal cellular processes such as mitochondrial metabolism and protein folding. While cancer cells have intrinsically higher levels of ROS than normal cells, ROS play an important role in cellular signaling. Slightly elevated ROS in cancer cells is crucial for promoting cell proliferation and activating survival pathways[11]. However, excessive ROS generation can be toxic and renders cancer cells more vulnerable to damage due to further oxidative stress induced by

some exogenous agents[12, 13]. Indeed, recent studies have found that ROS production partially mediates CDDP-induced apoptosis in cancer cells [14, 15]. Hence, it is necessary to develop effective agents that can induce oxidative stress to potentiate the anticancer effect of CDDP and facilitate its clinical application.

Andrographolide (AP) is a natural product isolated from *Andrographis paniculate*. AP has been used for the treatment of fever, diarrhoea, inflammation, and other infectious diseases in clinic without showing any obvious side effects[16–19]. Multiple studies have demonstrated that AP shows anti-cancer activities against various cancer cells both *in vitro* and *in vivo*[20–23]. It has also been reported that AP induces apoptosis in human colon cancer and lymphoma cells through ROS production while exerting little toxic effect on normal cells[23, 24]. Moreover, AP treatment led to a significant increase of ROS production in cancer cells as compared to normal cells; this discrepancy may underlie the selective cancer-killing ability of AP[24]. In this study, we intended to determine whether AP, a natural inducer of ROS, enhances the cytotoxicity of CDDP against colon cancer cells. Furthermore, we investigated the underlying mechanism of cell death triggered by combined treatment of colon cancer cells with AP and CDDP.

Materials And Methods

Cell viability assay

HCT116 and HT29 cells were seeded in a 96-well plate at a density of 8×10^3 cells/well and grown overnight. CDDP was dissolved in DMSO and diluted with 1640 medium to final concentrations of 2.5, 5, 10, 20, and 40 μM . Cells were incubated with CDDP or a combination of CDDP and AP (5 μM) for 24 h before MTT assay.

Cell cycle analysis

HCT116 cells were plated on a 6-well plate at a density of 3×10^5 cells/well and cultured for 24 h. Thereafter, the cells were treated with 5 μM AP, 15 μM CDDP, or a combination of AP and CDDP for 18 h. The DNA was labeled with propidium iodide (PI). Cell cycle analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, CA).

Apoptosis detection

HCT116 cells were plated on a 6-well plate at a density of 3×10^5 cells/well and cultured for 24 h. Afterwards, the cells were pre-treated with 5 mM NAC for 2 h, followed by treatment with 5 μM AP, 15 μM CDDP, or a combination of AP and CDDP for 24 h. Finally, cells were harvested, washed twice with PBS and then stained with Annexin V-FITC and PI. Apoptotic cells were detected using a FACSCalibur flow cytometer (BD Biosciences, CA).

Measurement of reactive oxygen species

Cellular ROS levels were measured by flow cytometer and fluorescence microscope. Briefly, HCT116 cells were plated on a 6-well plate at a density of 3×10^5 cells/well and cultured for 24 h. Thereafter, the cells were pre-treated with 5 mM NAC for 2 h, followed by treatment with 5 μ M AP, 15 μ M CDDP, or a combination of AP and CDDP for 2 h. Finally, cells were stained with 10 μ M DCFH-DA (Beyotime Biotech, China) at 37°C for 30 min and then subjected to fluorescence analysis using a FACSCalibur flow cytometer. Images were captured using Nikon fluorescence microscope equipped with a digital camera (Nikon, Japan).

Western blot analysis

HCT116 cells were seeded in a cell-culture plate at a density of 3×10^5 cells/well and grown in a humidified 5% CO₂ incubator at 37°C overnight. After being pre-treated with 5 mM NAC for 2 h, followed by incubation with 5 μ M AP, 15 μ M CDDP, or a combination of AP and CDDP along with DMSO vehicle for 24 h, cells were harvested, washed twice with PBS and lysed with the lysis buffer containing protease inhibitor cocktail. Cell lysates were then collected and centrifugated at 12000 g for 15 min at 4°C. Protein extracts were quantified using the Bradford method. Protein samples (20 μ g for each) were resolved on the SDS-PAGE and then transferred to a PVDF membrane (Millipore, IPVH00010). After being blocked with a 5% (w/v) fat-free milk blocking solution containing 0.1% Tween-20 dissolved in TBS, the membrane was incubated with primary antibodies against the target protein (1:1000 dilutions) overnight at 4°C. On the following day, the membrane was washed with TBST buffer three times and then incubated with horseradish peroxidase-conjugated secondary antibodies. The target protein was visualized by using ECL kit (Bio-Rad, Hercules, CA).

Cell transfection

HCT116 cells were plated in a 6-well plate at a density of 2×10^5 cells/well and cultured for 24 h. The cells were transfected with siRNA against ATF4 or a non-targeting control at a final concentration of 50 pmol mL⁻¹ using lipofectamine 2000 reagent (Invitrogen, CA). After 8 h of transfection, culture medium was replaced with the fresh medium and cells were incubated for an additional 36 h. Thereafter, the transfected cells were treated with 5 μ M AP or 15 μ M CDDP for 3 h and subjected to subsequent experiments. The oligonucleotides of siRNA were synthesized by GenePharma (Shanghai, China). siRNA against ATF4 were used in the study. Sequence: (sense: 5'-GCGUAGUUCGCUAAGGUGATT-3', antisense: 5'-UCACCUUAGCGAACUACGCTT-3').

Statistical analysis

All experiments were randomized and blinded. Data from 3 independent experiments is expressed as Mean \pm SEM. Statistical analysis was performed with GraphPad Prism 8.0 software (GraphPad, San Diego, CA, USA). Comparisons among more than two groups were conducted using one-way ANOVA followed by Dunnett's post hoc test, and comparison of multiple independent groups was performed by one-way ANOVA and non-parametric Kruskal–Wallis test followed by Dunn's post hoc test. The unpaired Student's t-test was carried out to compare the data between two groups. A p value < 0.05 was considered statistically significant.

Results

Combination of AP and CDDP exhibits synergistic anti-cancer effects

We first determined the effect of combined administration of AP and CDDP on viability of colon cancer cells. As shown in Figs. 1A-B, AP treatment alone caused no obvious changes in the viability of HCT116 and HT29 cells, whereas combination of CDDP and AP showed higher antiproliferative activities on the cancer cells than the single agent. We next investigated the underlying mechanisms of synergistic effect of AP and CDDP on HCT116 cells. To this end, we performed flow cytometric assays to comparatively analyze the cell cycle distribution among different groups. As illustrated in Figs. 1C-D, AP in combination with CDDP caused a marked increase in the proportion of cells in G2/M phase compared with AP or CDDP alone, as reflected by the increase of the proportion from 16.5% (CDDP) or 25.3% (AP) to 43.2% (combination of CDDP and AP). Consistent with the flow cytometry, western blot analysis revealed that AP markedly enlarged CDDP-induced decrease in the expression of cell cycle-related proteins MDM2, Cyclin B1 and CDC2 in HCT116 cells (Figs. 1E-F). All these data suggested that AP enhances CDDP-induced cell growth inhibition in HCT116 cells possibly through inducing a cell cycle arrest at G2/M phase.

Ap Potentiates Pro-apoptotic Effect Of Cddp On Hct116 Cells

To determine whether apoptosis mediates growth inhibition of colon cancer cells caused by the combined treatment, we examined pro-apoptotic effects of AP, CDDP and AP plus CDDP on HCT116 cells, respectively. As shown in Figs. 2A-B, combined treatment with AP and CDDP resulted in a significant increase in apoptosis rate as compared to AP or CDDP alone. Moreover, western blot analysis revealed that treatment with AP obviously enhanced cisplatin-induced activation of caspase-3 (cleaved caspase3), while reducing the expression level of Pro-Caspase3 (Figs. 2C-E). Together, these data indicated that combined treatment of AP and CDDP activates caspase-dependent apoptosis pathways, suggesting that AP treatment could promote cisplatin-induced apoptosis in HCT116 cells.

AP Potentiates Cisplatin-induced Apoptosis Via Ros Accumulation

Previous studies have shown that ROS generation could trigger cell apoptosis[25, 26]. Notably, AP has been found to induce oxidative stress mediated apoptosis via ROS generation in multiple cancer cell lines[23, 27, 28]. We, therefore, analyzed intracellular ROS generation in HCT116 cells by using 2',7'-dichlorofluorescein diacetate (DCFH-DA) staining. As depicted in Figs. 3A-B, treatment of HCT116 cells with AP or cisplatin alone led to a slight increase in ROS levels, while the ROS level was drastically increased in the cells treated with AP and cisplatin. Consistently, the above observations were reflected in the fluorescence intensity assay (Figs. 3C-D). To determine whether the ROS signal is involved in apoptosis induced by combined treatment of AP and CDDP, we pre-treated the cancer cells with NAC, a ROS inhibitor, and performed further studies. As shown in Figs. 3E-F, pretreatment of cells with 5 mM NAC prevented apoptosis in HCT116 cells caused by combined administration of AP and CDDP. Collectively, these results demonstrated that increased ROS levels contribute to cytotoxicity of the combined treatment against colon cancer cells.

ER stress contributes to apoptosis in colon cancer cells induced by the combination of AP and CDDP

Elevated ROS levels have been reported to induce apoptosis through several downstream pathways including endoplasmic reticulum (ER) stress-related pathway[29]. Indeed, ER stress acts as an important modulator in various pathologic conditions. Besides, activating ER stress progress usually contributing to cancer cell death by using therapeutic agents[30, 31]. Notably, AP could selectively kill cancer cells by activating ER stress[24]. Here, we sought to investigate whether ER stress contributes to cell apoptosis induced by combined treatment of AP and CDDP. For this purpose, we first examined the expression of ER stress-related proteins in HCT116 cells treated with AP and CDDP. As depicted in Figs. 4A-B, treatment of the cells with AP or CDDP for 3 h led to a slight increase in the expression level of p-EIF2 α and ATF4, while the expression of p-EIF2 α and ATF4 was dramatically increased in the cells treated with AP and CDDP for 3 h. Similarly, we found that combined treatment of AP and CDDP for 10 h resulted in a marked increase in the expression level of CHOP. We next investigated whether ER stress is involved in the apoptosis induced by the combined treatment. In the experiments, we knocked down the expression of ATF4 and then challenged the cells to AP and CDDP (Figs. 4C-D). As shown in Fig. 4E, silencing ATF4 led to an inhibition in the cell death following the combined treatment. Taken together, these data indicated that ER stress plays a key role in apoptosis of the cancer cells treated with AP and CDDP.

The combined treatment inhibits STAT3 phosphorylation in colon cancer cells

Signal transducer and activator of transcription 3 (STAT3) is an oncogenic transcription factor that is continuously activated in various types of solid tumors, including colon cancer[32–34]. It has been reported that AP improves chemosensitivity of cancer cells to doxorubicin by inhibiting JAK/STAT3 pathway[35]. We further examined whether combined treatment of AP and CDDP modulates STAT3 activation in HCT116 cells. As shown in Figs. 5A-B, compared with the control, cells treated with AP or

CDDP displayed a slightly reduced expression of p-STAT3, while a marked decrease in the expression level of p-STAT3 was detected in cells treated with AP and CDDP. Notably, pretreatment of cells with 5 mM NAC relieved the reduced expression of p-STAT3 caused by the combined treatment (Figs. 5C-D). These data indicated that the combined treatment inhibits STAT3 phosphorylation possibly through ROS generation. Altogether, these findings suggested a unique approach for targeting STAT3 signaling pathway by ROS-inducing agents (AP combined with CDDP).

The combined treatment inhibits xenograft tumor growth of HCT116 cells

To determine *in vivo* anti-cancer effect of the combined treatment, we performed functional assays on a subcutaneous xenograft tumor model of HCT116 cells. As illustrated in Figs. 6A-C, combined administration of AP and CDDP led to an effective inhibition in the growth of tumor xenograft. Clearly, the combined treatment showed a stronger inhibitory effect on tumor weight and volume. Further studies revealed that the combined treatment significantly increased the level of lipid peroxidation product (MDA), a ROS marker, comparing with single treatment group (Fig. 6D). Moreover, immunohistochemical assays identified a marked decrease in the expression of Ki-67 as well as an increase in the expression of cleaved caspase3 in the xenograft tumor model administered with AP and CDDP (Fig. 6E). Taken together, these results demonstrated that AP synergizes with CDDP in inhibiting the tumor growth *in vivo* possibly by increasing ROS levels.

Discussion

Colon cancer is one of the leading causes of cancer-related deaths and continues to be a global health problem. While great advances have been made in the treatment of colon cancer using surgery, 40-50% of patients with colon cancer who underwent potentially curable surgery eventually relapse or die of metastatic disease[36, 37]. Cisplatin (CDDP) is commonly used as first-line chemotherapy against advanced colon cancer, but its clinical benefits are limited by the side toxicity and drug resistance[5]. Therefore, the development of new drugs and/or new therapeutic combination of CDDP against colon cancer is urgently needed. Recently, increasing attention has been paid to the combination of CDDP and phytochemicals including AP because of its low toxic effect and biological activity[38–40]. We, therefore, sought to investigate whether AP enhances the anticancer effect of CDDP on colon cancer both *in vitro* and *in vivo*. In the present study, we showed that AP increases cellular ROS levels and potentiates the cytotoxic effects of CDDP on colon cancer cells. Furthermore, AP synergized with CDDP in inducing apoptosis through activating ROS-dependent ER stress and inhibiting STAT3 signaling pathway (summarized in Fig. 7).

Compared with normal cells, cancer cells appear to be more vulnerable to damage resulting from excessive production of ROS by some exogenous agents[41, 42]. A growing number of studies have found that increased ROS production may lead to growth inhibition and apoptosis in colon cancer cells[43, 44]. Thus, manipulation of ROS levels through redox modulation could be a promising strategy to selectively kill cancer cells without causing obvious toxicity to normal cells. Increased ROS can cause

oxidative stress, leading to the destabilization of mitochondria and eventually apoptosis. Apoptotic cells exhibit typical apoptotic features, including caspase activation, DNA fragmentation and apoptotic body formation[45]. Constitutive STAT3 activation is commonly identified in colon cancer and associated with poor survival of colon cancer patients[46, 47]. It has been suggested that inhibition of STAT3 signaling pathway by pharmacological agents may potentially become a new therapeutic strategy for cancer [34].

AP is a diterpenoid lactone that has a wide range of pharmacological activities, such as antiviral, anti-inflammatory, immunomodulatory, and antioxidant effects[16, 17]. In addition, AP exhibits an excellent anti-tumor effect on various tumor cells[20, 21, 28]. Recent studies have shown that AP induces apoptosis in colon cancer cells through activating ROS-dependent ER stress pathway [24]. Moreover, AP can potentiate chemosensitivity of human colon cancer cells to doxorubicin through inhibiting the STAT3 pathway[35]. Herein, we identified a role of AP in sensitizing colon cancer cells to CDDP treatment. Strikingly, a combination of AP and CDDP exerted a significant pro-apoptotic effect on colon cancer cells through ROS-mediated ER stress and STAT3 inhibition. In the meantime, combined treatment with AP and CDDP led to a marked increase in the expression level of p-EIF2 α and ATF4. Further studies demonstrated that cellular ROS mediates the above effects. Besides, we observed that the expression of ER stress marker CHOP was significantly increased in colon cancer cells treated with AP and CDDP. Furthermore, we demonstrated that blocking ER stress through CHOP knockdown significantly relieved apoptosis of the cancer cells induced by the combined treatment, whereas activating ER stress promoted the apoptosis. Finally, we showed that a combination of AP and CDDP caused ROS-mediated STAT3 inhibition in colon cancer cells. Hence, the present study provided new insights into the underlying mechanisms of effects of the combined treatment on colon cancer.

In conclusion, we demonstrated that AP enhances the anti-tumor effect of CDDP on colon cancer cells by increasing ROS production, while combined treatment of AP and CDDP induces apoptosis through ROS-mediated ER stress and inhibition of STAT3 signaling pathway. These findings contribute to a better understanding of mechanisms underlying synergistic effects of AP and CDDP on the treatment of colon cancer.

Declarations

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Author Contributions

This work was carried out in collaboration among all authors. H H and WL C performed the research. H H, WL C and CJ H designed the research. QP W, WL C and CB L analysed the data. H H, CB L and CJ H wrote the paper. All authors have read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figures

Figure 1

Combination of Andrographolide (AP) and Cisplatin (CDDP) exhibited synergistic anticancer effect in colon cancer.

(A-B) AP enhances CDDP-induced growth inhibition in colon cancer cells. Briefly, HCT116 and HT29 cells were treated with AP and cisplatin for 24 h, cell viability was determined by MTT assay. (C-D) AP enhances cisplatin-induced cell cycle arrest in HCT116 cells. Cells were treatment for 18 h, and then stained with PI solution after fixation by 70% ethanol. Cell cycle distribution was analyzed by flow

cytometric analysis. Representative flow cytometric graph shown in panel (D) [n = 3; *P < 0.05 and **P < 0.01 compared to control]. (E) Expression of G2/M cell cycle relative proteins CDC2, Cyclin B1 and MDM2 were determined by western blot after treatment for 18 h in HCT116 cells. GAPDH was used as internal control. (F) Western blot results from (E) were calculated and represented as the percent of control [n = 3; *P < 0.05 and **P < 0.01 compared to control].

Figure 2

AP enhances CDDP-induced cell apoptosis in HCT116 cells.

(A-B) AP enhances CDDP-induced apoptosis in HCT116 cells. Cells were collected after treatment for 24 h and then stained with Annexin V and PI. Cell apoptosis rate was analyzed by flow cytometric analysis. The percentage of apoptotic cells in the treatment groups was calculated. Representative flow cytometric graph shown in panel (B) [n = 3; *P < 0.05 and ***P < 0.001 compared to control]. (C) Western blot analysis of expression levels of Pro-Caspase3 and Cleaved-Caspase3 in HCT116 cells. (D-E) Western blot results from (C) were calculated and represented as the percent of control [n = 3; *P < 0.05 and **P < 0.01 compared to control].

Figure 3

AP potentiates CDDP-induced apoptosis by ROS accumulation in HCT116 cells.

(A-B) Intracellular ROS generation induced by AP and/or CDDP were measured by staining with DCFH-DA (10 mM) and flow cytometry analysis. Representative flow cytometric graph shown in panel (b) [n = 3; *P < 0.05 and ***P < 0.001 compared to control]. (C-D) Fluorescence images of DCFH-DA-stained cells (green) were captured [scale bar = 10 μm]. Quantification of ROS levels in HCT116 cells is shown in panel (D). (E-F) HCT116 cells were pre-incubated with or without 5 mM NAC for 2 h before combined treatment. Percentage of cell apoptosis was determined by Annexin-V/PI staining and flow cytometry. Representative flow cytometric graph shown in panel (F) [n = 3; ***P < 0.001 compared to control, ##P < 0.01 compared to CDDPAP group].

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Figure 4

AP potentiates CDDP-induced apoptosis depending on ER stress in HCT116.

(A) Combined treatment increased the expression of p-EIF2 α , ATF4 and CHOP proteins. HCT116 cells were incubated with AP and/or CDDP for 3 h or 10 h and then the protein levels of p-EIF2 α , ATF4 and CHOP were analyzed by western blotting. (B) Western blot results from (A) were calculated and represented as the percent of control [n = 3; *P < 0.05, **P < 0.01 and ***P < 0.001 compared to control]. (C) Western blot analysis of ATF4 protein following transfection of HCT116 cells with siRNA against ATF4 before combination treatment. (D) Western blot results from (C) were calculated and represented as the percent of control [n = 3; **P < 0.01]. (E) Assessment of Annexin V/PI staining positive cells following knockdown of ATF4 and exposure to CDDP plus AP treatment [n = 3; **P < 0.01].

Figure 5

Combined treatment inhibit STAT3 phosphorylation in HCT116 cells.

(A) Combined treatment decreased the expression of p-STAT3 and STAT3 protein. HCT116 cells were incubated with AP and/or CDDP for 12 h and then protein level of p-STAT3 was analyzed by western blotting. STAT3 was used as internal control. (B) Western blot results from (A) were calculated and represented as the percent of control [n = 3; *P < 0.05, **P < 0.01 and ***P < 0.001 compared to control]. (C) HCT116 cells were pre-incubated with or without 5 mM NAC before exposure to CDDP plus AP for 12 h, the expression of p-STAT3 and STAT3 were detected by western blot. STAT3 was used as internal control. (D) Western blot results from (C) were calculated and represented as the percent of control [n = 3; *P < 0.05 and **P < 0.01 compared to control].

Figure 6

Combined treatment of AP and CDDP inhibits HCT116 xenograft tumor growth *in vivo*.

(A-B) Combined treatment of AP and CDDP significantly inhibited tumor volume and tumor weight (C) of HCT116 human colon cancer xenografts in nude mice. [n = 3; *P< 0.05, **P< 0.01 and ***P< 0.001 compared to control]. (D) The levels of MDA in the tumor tissues. [n = 3; **P< 0.01 and ***P< 0.001 compared to control]. (E) The levels of Ki-67 and Cleaved caspase3 in tumor tissues were analyzed by immunohistochemical analysis.

Figure 7

Schematic illustration of the ROS-ER stress/STAT3 signalling axis in combined treatment of CDDP and AP induced apoptosis in colon cancer.